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Transcriptional Responses to Gibberellin and Abscisic Acid in Barley Aleurone

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Abstract

Cereal aleurone has been established as a model system to investigate giberrellin (GA) and abscisic acid (ABA) responses. Using Barley 1 GeneChip, we examined the mRNA accumulation of over 22 000 genes in de-embryonated barley aleurone treated with GA and ABA. We observed that 1 328 genes had more than a threefold change in response to GA treatment, whereas 206 genes had a more than threefold change in response to ABA treatment. Interestingly, approximately 2.5-fold more genes were up-regulated than down-regulated by ABA. Eighty-three genes were differentially regulated by both GA and ABA. Most of the genes were subject to antagonistic regulation by ABA and GA, particularly for genes related to seed maturation and germination, such as genes encoding late embryogenesis abundant proteins and storage mobilization enzymes. This supports the antagonistic roles of GA and ABA in seed maturation and seed germination. Interestingly, we observed that a significant percentage of the genes were coordinately regulated by both GA and ABA. Some GA-responsive genes encoded proteins involved in ethylene, jasmonate, brassinosteroid and auxin metabolic and signaling transduction pathways, suggesting their potential interaction with the GA response. We also identified a group of transcription factor genes, such as MYB and Homeobox genes, that were differentially regulated by GA. In addition, a number of GA- and/or ABA-responsive genes encoded components potentially involved in GA and ABA signal transduction pathway. Overall, the present study provides a comprehensive and global view of transcript expression accompanying the GA and ABA response in barley aleurone and identifies a group of genes with potential regulatory functions in GA- and ABA-signaling pathways for future functional validation.

Key words: abscisic acid; barley; gene regulation; gibberellin; microarray; signal transduction.

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Gibberellins (GA) and abscisic acid (ABA) are two phytohormones regulating many agronomical important aspects of plant development and the physiological response to environmental stresses. It is believed that GA and ABA are the major factors regulating developmental transition from seed development to germination. A rise in ABA levels during embryogenesis is often correlated with deposition of storage nutrients, the acquisition of desiccation tolerance, and seed dormancy. Breaking of seed dormancy is frequently associated with a decrease in ABA levels. In contrast, application of exogenous GA often promotes seed germination. Treatments promoting seed germination, such as cold and light, are often correlated with an increase in endogenous GA (Yamaguchi et al. 1998). It is proposed that the GA/ABA balance governs the maturation versus germination pathway (White et al. 2000).

During cereal grain germination and seedling growth, GA is

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produced in the embryo and is relocated into the aleurone tissues, where it activates the production of many hydrolytic enzymes. The hydrolytic enzymes are further secreted into the endosperm to mobilize storage reserves for seedling growth. The production and secretion of the hydrolytic enzymes in aleurone tissues are regulated by GA. It is believed that the deembryonated cereal aleurone does not synthesize endogenous biological active GAs (Kaneko et al. 2003), but respond strongly to GA and ABA treatment in producing hydrolytic enzymes (Chrispeels and Varner 1967). In addition, isolated aleurone layers are composed of one living cell type, and should respond uniformly to GA and ABA treatments. Therefore, the isolated deembryonated cereal aleurone system provides an excellent system for the investigation of the mode of action of GA and ABA and has been used to successfully identify a large number of components in the GA and ABA response pathways over the past decades (Lovegrove and Hooley 2000; Sun and Gubler 2004).

A number of components are likely to be involved in the GA-induced production of α-amylase. GAMYB, a transcription factor, is highly induced by GA, and regulates the expression of the gene encoding α-amylase (Gubler et al. 1995). Calcium (Gilroy and Jones 1992), calmodulin (Penson et al. 1996), GTP-binding proteins (Ashikari et al. 1999), kinases (Gomez-Cadenas et al. 1999), and protein phosphatases (Kuo et al. 1996) have been suggested to be involved in the pathway. Recently, a DELLA protein and an F-box protein were discovered to be involved in GA signal transduction (Sasaki et al. 2003). Phosphorylation of the DELLA protein is required for the interaction of the two proteins and activation of the 26S proteasome to degrade the DELLA protein (Gomi et al. 2004). The DELLA proteins function as repressors in the GA signaling pathway, and are known to be responsible for several dwarf or slender mutants, depending on the site of the mutation (Peng et al. 1999; Chandler et al. 2002; Dill et al. 2004). The ubiquitin/26S proteasome-mediated protein degradation of DELLA proteins is likely to be conserved in the GA signal transduction pathways (Tyler et al. 2004). In addition, a GA receptor has been identified recently (Ueguchi-Tanaka et al. 2005).

Research also shows that expression of many genes can be differentially regulated by ABA. Two Basic-leucine zipper (bZIP) transcriptional factors, namely HvABI5 and HvVP1, have been reported to be involved in ABA-induced *HVA1* and *HVA22* gene expression, but not in GA-induced and ABA-suppressed α-amylase gene expression, in aleurone (Casaretto and Ho 2003). However, HvSPY functions as a positive regulator of ABA-induced dehydrin gene expression and a negative regulator of GA-induced α-amylase gene expression (Lang et al. 1998). The protein kinase pKABA1, which is up-regulated by GA but down-regulated by ABA, suppresses GA-induced α-amylase gene expression, but has little effect on ABA-induced *HVA1* expression (Gomez-Cadenas et al. 1999).

However, the GA and ABA signal transduction pathways and their interaction are still far from being clearly understood. For example, calcium has been demonstrated to be involved in these processes (Gilroy and Jones 1992), but no gene has been identified as responsible. In the present study, using Barley 1 GeneChip (Close et al. 2004), we analyzed the mRNA accumulation of over 22 000 genes in barley aleurone tissues treated with GA and ABA. The study provides a global picture of the genome-wide transcript profile in barley aleurone in response to GA and ABA.

Results and Discussion

Transcript profiles of genes regulated by GA and ABA

The timing and concentration of ABA and GA selected for the treatments were based on prior reports (Nolan and Ho 1988) and our analysis of α-amylase activity and its mRNA accumulation in response to the treatments. In the present study, we used 1 μmol/L GA_3 and 50 μmol/L ABA to treat barley aleurone tissues for 15 h. With the concentrations used, α-amylase activity was increased 19-fold following GA treatment and decreased threefold following ABA treatment. Compared with GA treatment, α-amylase activity was decreased by 88% in the GA plus ABA treatment group (Figure 1A). The GA treatment

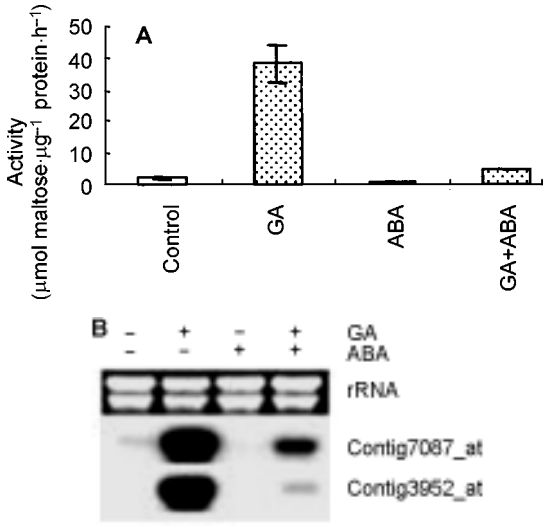

Figure 1. α-Amylase expression in the treated aleurone tissues used for microarray experiments. The aleurone tissue from half-grains of barley cv. Himalaya were incubated with or without gibberellic acid (GA_3) at 10^{-6} mol/L or abscisic acid (ABA) at 5×10^{-5} mol/L. Samples were incubated for 15 h.

(A) α-Amylase activity.

(B) Northern blotting of two α-amylase genes (+, with the hormone; –, without the hormone).

also markedly induced mRNA accumulation of genes encoding a high PI amylase (Contig3952_at) and a low PI amylase (Contig7087_at), whereas ABA significantly suppressed GA induction (Figure 1B). All the data indicate that the treatments applied under the present study conditions were effective and led to a significant change in barley aleurone.

Using the MicroArraySuite (MAS), we detected 11 483 genes in the control group, 11 225 genes in the GA treatment group, and 12 204 genes in the ABA treatment group. Based on 22 792 probe sets on the chip, approximately 50% of the genes were expressed above the detection limit. Among these, 9 993 genes were expressed in all groups (control and GA, ABA treatment; Figure 2). Fewer genes were specifically expressed in the control group (224), the ABA treatment group (800), or the GA treatment group (629).

To further assess transcriptional profiles following GA and ABA treatments, significance analysis of microarrays (SAM) (False Discovery Rate, 1%, median) was used to identify the genes whose steady mRNA levels changed significantly in response to GA or ABA treatment. In all, 4 611 genes exhibited a significant response to GA treatment, whereas 1 572 genes were found to respond significantly to ABA treatment. The number of GA-responsive genes was approximately threefold that of the ABA-responsive genes. Among the 4 611 GA-responsive genes, 2 076 genes were up-regulated, whereas 2 535 were down-regulated. Among the ABA-responsive genes, 1 133 were up-regulated, whereas 439 were down-regulated. Obviously, more genes were up-regulated than down-regulated by ABA treatment, and fewer genes were up-regulated than down-regulated by GA. Figure 3 shows the distribution of genes in terms of changes in their expression. The majority of GA- and ABA-responsive genes showed a less than threefold change in their mRNA accumulation. However, a higher percentage of GA-responsive genes (30%) changed more than threefold compared with the ABA-responsive genes (13%). In all, 1 328 GA-responsive genes and 206 ABA-responsive genes showed a greater than threefold change. Of the 1 328 GA-responsive genes, 683 were up-regulated, whereas 645 were down regulated. In addition, 147 and 59 genes were identified as being up- and down-regulated in response to ABA, respectively. To limit our discussion to the genes showing more marked responses to GA and ABA treatments, the following describes differentially regulated genes showing a more than threefold change and statistical significance, unless specified otherwise.

Differential regulation of the genes encoding hydrolytic enzymes

One of the most important functions of barley aleurone is to produce and secrete hydrolytic enzymes for mobilizing the reserve stored in the endosperm to support seedling growth. The mobilization of the storage reserve in cereal endosperms is strongly induced by GA and suppressed by ABA (Reviewed by Fincher 1989). In the present experiment, a large number of genes encoding hydrolase were up-regulated by GA and down-regulated by ABA. The genes encoding hydrolase acting on the glycosyl bond, peptide bond, and ester bond were particularly highly represented in the group of GA up-regulated genes (Figure 4). The GeneChip contains 1.31% of probe sets representing

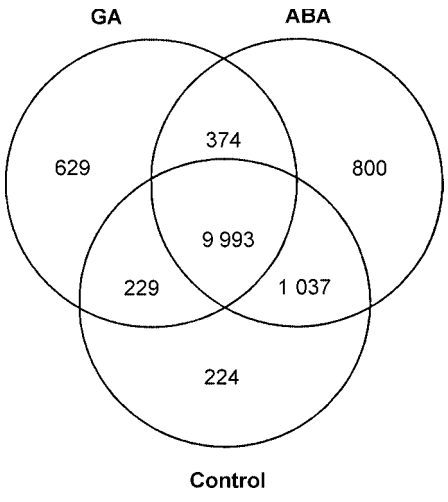

Figure 2. Venn diagram of the expressed genes in giberrellin (GA)- and abscisic acid (ABA)-treated groups and control.

The numbers in the areas represent the numbers of genes detectable in the denoted treatments.

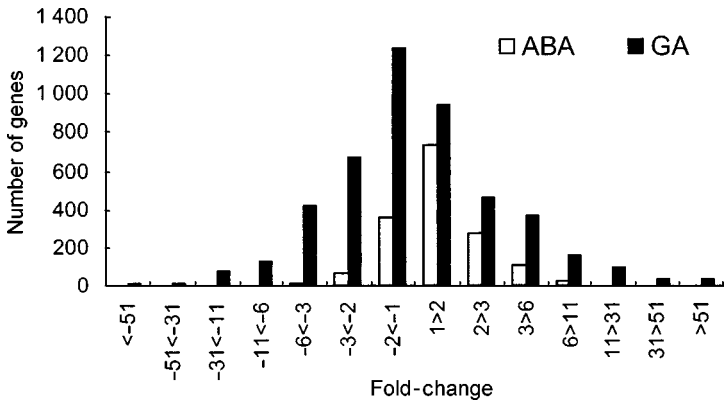

Figure 3. Distribution of genes differentially regulated by giberrellin (GA) and abscisic acid (ABA).

Significance analysis of microarrays (SAM) was used to identify the genes significantly responsive to GA or ABA treatment. The number of the genes (*y*-axis) with each denoted range of fold changes (*x*-axis) is shown. Both 1 and –1 indicate no change in response to GA or ABA.

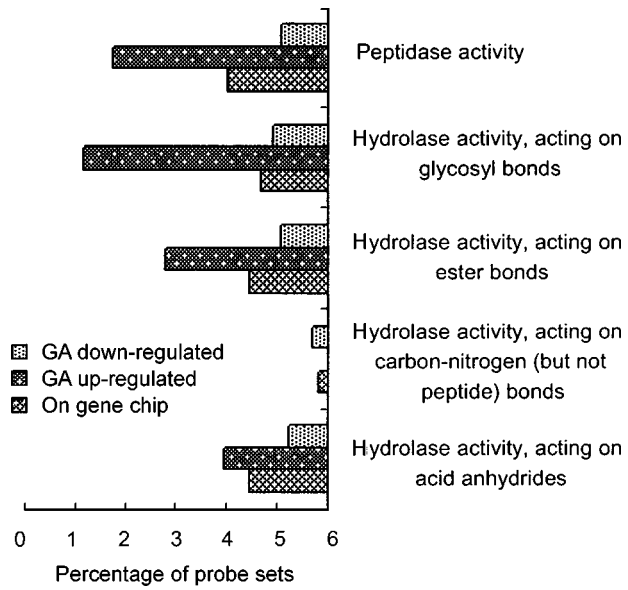

Figure 4. Representation of the genes encoding hydrolytic enzymes in giberrellin (GA)-responsive genes.

The percentage of probe sets representing proteins with denoted hydrolytic activities (*y*-axis) in GA-down-regulated, -up-regulated and all examined probe sets are indicated along the *x*-axis. Composition and Gene Ontology (GO) terms were assigned by the FuncExpression in BarleyBase

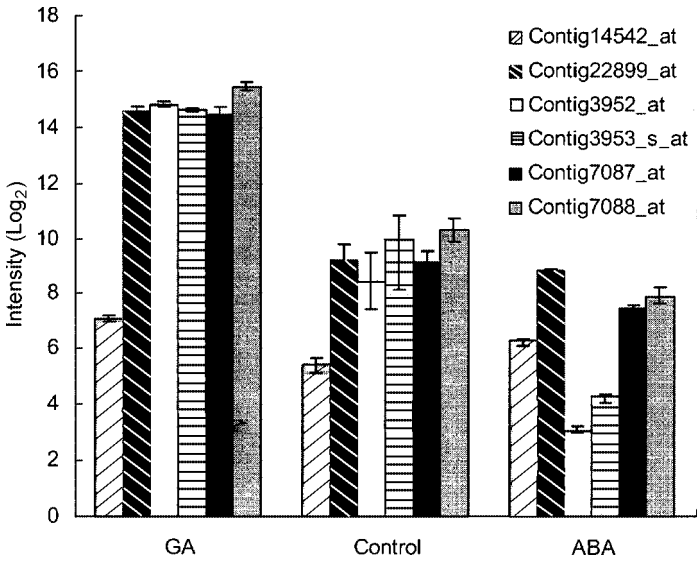

Figure 5. Expression of α-amylase genes in response to gibberellin (GA) and abscisic acid (ABA) treatments.

Average signal intensities of six probe sets representing amylase genes are indicated. The intensity ranges of the three replicates are marked.

hydrolases acting on the glycosyl bond, 1.98% of those acting on the peptide bond, and 1.54% of those acting on the ester bond however. The GA-up-regulated genes contains 4.83%, 4.35%, and 3.22% of probe sets in the three categories, respectively, whereas the GA-down-regulated genes contain 1.09%, 0.93%, and 0.93%, respectively. Therefore, the probe sets were over-represented for the up-regulated genes and under-represented for the down-regulated genes. The hydrolytic enzyme genes were preferentially induced by GA. Table 1 lists the genes differentially regulated by GA or ABA that encode hydrolytic enzymes for the degradation of polysaccharides, proteins, nucleic acids, and lipids.

Forty-two genes differentially regulated by GA or ABA were involved in polysaccharide degradation (Table 1). Thirty-two of the 42 genes were up-regulated by GA treatment. Starch is the most abundant storage macromolecule in barley endosperms. Mobilization of the starch in the endosperm provides the major energy resource for early seedling growth. The actions of α-amylases, β-amylases, debranching enzymes, limit dextrinase, and α-glucosidases are required to mobilize starch to glucosyl residues. We observed that all the six α-amylase genes on the GeneChip were up-regulated by three- to 80-fold (Table 1; Figure 5). One limit dextrinase gene and two α-glucosidase genes were also up-regulated by GA. β-Amylase is required for starch mobilization during seed germination and early seedling growth. Interestingly, none of the seven β-amylase genes on the GeneChip showed more than a 1.6-fold expression change in response to GA or ABA. The seven genes were expressed at undetectable or very low levels (data not shown). It has been shown that β-amylase is synthesized exclusively in the starchy endosperm during seed maturation and stored in the endosperm tissues rather than in the aleurone after the initiation of germination (Lauriere et al. 1986; Kreis et al. 1987). Thus, two distinct regulatory programs are used to produce α- and β-amylases for starch mobilization. The β-amylase used in starch degradation during seed germination and early seedling growth is likely to be synthesized during seed maturation. The functional and evolutionary implications of using different regulatory programs to synthesize α- and β-amylases remains to be explored. Conversely, four α-amylase genes, which were shown to be up-regulated by GA, were down-regulated three- to 50-fold by ABA.

The cell wall constitutes a critical physical barrier preventing the hydrolytic enzymes in aleurone from reaching storage reserves in starchy endosperms, because the enzymes cannot move freely across the cell walls of endosperm (Fincher and Stone 1986). Depolymerization of

Table 1. GA and ABA differentially regulated genes for degradation of polysaccharides, proteins, nucleic acids and lipids

Barley 1 probe set ID	Fold-change (GA)	Fold-change (ABA)	Function annotation
Contig14542_at	3.13	1.85	Alpha-amylase (EC3.2.1.1)
Contig3952_at	83.30	–41.48	Alpha-amylase (EC3.2.1.1)
Contig22899_at	40.11	–1.31	Alpha-amylase (EC3.2.1.1)
Contig7087_at	39.83	–3.06	Alpha-amylase (EC3.2.1.1)
Contig7088_at	35.07	–5.38	Alpha-amylase (EC3.2.1.1)
Contig3953_s_at	25.18	–49.80	Alpha-amylase (EC3.2.1.1)
Contig15045_at	–3.25	2.46	Alpha-galactosidase (EC 3.2.1.22)
Contig11583_at	86.43	–1.82	Beta-galactosidase
Contig7937_s_at	18.73	–1.37	Alpha-glucosidase (EC 3.2.1.20)
Contig7938_at	9.24	2.22	Alpha-glucosidase (EC 3.2.1.20)
Contig2736_s_at	–4.08	1.87	Beta-glucosidase
Contig24491_at	–9.16	1.41	Beta-glucosidase
Contig10477_at	–1.07	4.22	Glucan endo-1,3-beta-D-glucosidase
Contig7032_at	4.70	–1.04	Alpha-*L*-arabinofuranosidase
Contig20715_at	7.58	4.86	Glucanase
Contig2834_at	14.40	–54.40	Beta glucanase
Contig17372_at	32.28	–2.46	Beta-1,3-glucanase
Contig13838_at	–8.76	1.15	Endo-beta-1,4-glucanase
HVSMEn0019D12r2_s_at	3.31	1.03	Endo-1,4-beta-glucanase
HU14A02u_at	5.14	1.59	Licheninase (EC 3.2.1.73)
Contig4970_at	4.18	–1.52	Beta-*D*-glucan exohydrolase
Contig5703_at	15.24	–3.90	Beta-*D*-xylosidase
Contig8591_at	12.26	–1.35	Beta-xylosidase
Contig13674_at	176.74	–1.31	Beta-*D*-xylosidase
Contig4470_s_at	88.41	–1.11	Extracellular invertase
Contig7811_s_at	5.82	1.70	Cell wall invertase
Contig6539_s_at	2.65	3.63	Cell wall invertase
Contig14993_at	–7.02	2.11	Vacuolar acid invertase
Contig11648_at	27.25	–1.86	Limit dextrinase
Contig14498_at	4.47	–1.05	Chitinase (EC 3.2.1.14)
Contig2990_at	–1.27	5.58	Chitinase (EC 3.2.1.14)
Contig9060_at	4.30	1.33	Chitinase (EC 3.2.1.14)
Contig5995_at	–4.30	3.51	Chitinase (EC 3.2.1.14)
Contig13792_s_at	113.82	–7.23	1,4-Beta-*D* xylan xylanohydrolase
Contig12475_at	22.26	–1.02	Polygalacturonase
Contig16010_at	73.04	–16.22	1,4-Beta-xylanase
Contig7337_at	4.46	2.29	Endoxyloglucan transferase
Contig11243_at	8.09	–1.24	Polygalacturonase (pectinase)
Contig12893_at	–3.43	–1.30	Polygalacturonase
Contig13013_at	8.16	–4.52	Polygalacturonase
HE01I24u_s_at	3.13	1.42	Xyloglucan endotransglycosylase
Contig2672_at	8.70	1.69	Xyloglucan endotransglycosylase
Contig9219_at	3.18	–1.44	Carboxypeptidase
HV_CEa0009O07r2_s_at	3.23	–1.09	Carboxypeptidase
Contig600_at	5.74	–1.20	Carboxypeptidase
Contig682_s_at	4.61	–1.40	Carboxypeptidase
HA16L09r_s_at	3.69	–1.09	Carboxypeptidase
Contig6685_at	6.17	–1.22	Carboxypeptidase
Contig6686_s_at	7.13	–1.94	Carboxypeptidase
Contig86_at	5.86	–1.88	Cysteine protease

Table 1 (continued).

Barley 1 probe set ID	Fold-change (GA)	Fold-change (ABA)	Function annotation
Contig5510_s_at	6.56	–1.13	Cysteine protease
Contig11268_at	3.67	1.53	Cysteine protease
Contig17638_at	9.81	–1.03	Cysteine protease
U19359_s_at	51.89	–50.43	Cysteine protease
Contig5278_at	176.42	–3.18	Cysteine protease
Contig5281_at	12.29	–4.54	Cysteine protease
Contig2555_at	4.80	–1.04	Cysteine protease
HVSMEn0023O21f_s_at	5.01	–1.30	Cysteine protease
Contig2556_s_at	6.68	–1.31	Cysteine protease
Contig2403_at	6.07	–1.30	Cysteine protease
Contig3900_at	7.86	–3.52	Cysteine protease
Contig3901_s_at	9.04	–9.75	Cysteine protease
rbaal21f05_s_at	5.38	–7.49	Cysteine protease
Contig6777_at	4.78	–1.30	Cysteine protease
Contig2680_at	3.04	–1.12	Cysteine protease
Contig2683_s_at	9.71	1.23	Cysteine protease
Contig2681_at	8.09	–1.13	Cysteine protease
Contig2401_at	3.26	1.13	Cysteine protease
Contig2402_s_at	3.13	1.02	Cysteine protease
HVSMEn0005A13f_s_at	3.25	1.08	Cysteine protease
Contig8854_at	–6.43	1.04	Serine protease
EBed02_SQ002_E18_s_at	–4.69	1.24	Serine protease
Contig6897_at	4.08	–1.11	Serine protease
HVSMEl0010A17f_s_at	3.33	–1.22	Serine protease
Contig7202_s_at	11.66	1.06	Serine protease
Contig25510_at	–3.27	1.26	Matrix metalloproteinase
Contig9418_at	–3.02	–1.44	Aspartyl protease
Contig20999_at	–3.77	–1.20	Acyl-peptide hydrolase
Contig2354_at	1.38	3.30	Aspartic protease
Contig4113_at	49.59	–2.83	Endonuclease
Contig4111_at	12.41	1.60	Endonuclease
Contig4112_at	5.78	–1.05	Endonuclease
HD13B05r_s_at	16.44	–1.02	Ribonuclease 1 (RNS1)
Contig3691_at	160.88	–2.97	Ribonuclease 1 (RNS1)
Contig7478_at	4.89	1.12	Ribonuclease 2 (RNS2)
Contig14247_at	–3.15	1.58	Exodeoxyribonuclease
Contig20457_at	3.25	1.39	3'(2'),5'-bisphosphate nucleotidase
HA11O05u_s_at	3.95	1.38	3'(2'),5'-bisphosphate nucleotidase
HA11O05u_at	5.45	1.43	3'(2'),5'-bisphosphate nucleotidase
Contig18370_at	218.39	–1.72	Phosphoesterase family protein
Contig8049_at	7.53	1.07	Glycerophosphoryl diester Phosphodiesterase
Contig16494_s_at	3.61	1.29	Glycerophosphoryl diester Phosphodiesterase
EBro08_SQ004_P13_at	3.39	1.42	Triacylglycerol lipase
Contig20537_at	–1.72	5.11	Triacylglycerol lipase
Contig19422_at	28.23	1.18	Triacylglycerol lipase

β-glucan and arabinoxylan, the major components in the cell wall, plays an important role at the beginning of storage mobilization (Taiz and Jones 1970; Taiz and Honigman 1976). In the present study, we observed that four of five GA-responsive β-glucanase genes were up-regulated three- to 30-fold by GA (Table 1). Although the information on arabinoxylan degradation is not very clear at present, an arabinofuranosidase gene (Contig7032_at) was observed to be up-regulated 4.7-fold by GA in the present study. This result is consistent with previous observations that GA induces α-arabinofuranosidase activity (Taiz and Honigman 1976). In the ABA treatment group, one β-glucanase gene (Contig2834_at), which was a GA-up-regulated gene, was markedly down-regulated 55-fold (Table 1).

In the present study, we observed (Table 1) that 21 probe sets representing cysteine protease genes were differentially regulated by GA. Interestingly, all 21 probe sets were up-regulated by GA over a range of three- to 176-fold. In addition, seven carboxypeptide genes and three serine protease genes were up-regulated by GA. We also observed that six of seven GA-responsive nuclease genes and all three GA-responsive nuclotidase genes (Table 1) were up-regulated by GA from three- to 160-fold. One GA-responsive exodeoxyribonuclease gene (Contig14247_at) was down-regulated threefold by GA. There were significant levels of residual RNA and DNA remaining in the non-living cells of the starchy endosperm in mature barley grain (McFadden et al. 1988). The GA-induced production of nuclease and nucletidase may be to mobilize the nucleic acid remaining in the starchy endosperm. In addition, two triacylglycerol lipase genes (EBro08_SQ004_P13_at and Contig19422_at) were up regulated by GA in the present study. Following ABA treatment, among the 21 GA-up-regulated cysteine protease genes, six were down-regulated three- to 10-fold by ABA. It is consistent to the previous reports that barley aleurone also synthesizes a complex group of proteases, nucleases, nucletidases, and lipases, along with α-amylase, in response to GA treatment (Chrispeels and Varner 1967; Mikola 1983; Koehler and Ho 1990). Carboxypeptidase and cysteine endopeptidase were also identified in germinated barley seeds (Mikola 1983).

Taken together, our microarray data provided strong evidence on a genome level that the transcriptional expression of many hydrolytic enzymes is preferentially up-regulated by GA, but suppressed by ABA, in barley aleurone tissues. Many of the GA-up-regulated hydrolytic enzymes may function to mobilize the storage reserve, including polysaccharides, proteins, nucleic acids, and even lipids. However, some hydrolase genes in barley aleurone may be involved in other biological processes rather than storage mobilization. For example, we observed that two chitinase genes were up-regulated by GA. There is no chitin substrate for the enzymes in barley grain. Chitinases were proposed to function as potent inhibitors of fungal growth (Schlumbaum et al. 1986; Jacobsen et al. 1990). Therefore, it is plausible that the chitinase may function to defend against invasion of pathogens during seed germination. We also observed that some hydrolytic enzymes were down-regulated by GA treatment. For example, the genes encoding two serine proteases, an aspartyl protease, an acyl-peptide hydrolase, and a matrix metalloproteinase were down-regulated by GA treatment. The biological implication of the GA down-regulation of hydrolytic enzymes remains to be determined.

Differential regulation of transcription factor genes

In the present study, we observed that 70 putative transcription factors or transcriptional regulators were differentially regulated by GA and/or ABA (Table 2). Eight *MYB* genes were differentially regulated by GA; only Contig13879_at was down-regulated (12.5-fold). The probe sets, X87690_s_at and HS18K19u_s_at, represent an extensively studied *GAMYB* (Gubler et al. 1995). Both probe sets were up-regulated approximately 4.5-fold by GA. The GAMYB transactivates a number of GA-responsive genes encoding hydrolytic enzymes, including α-amylase in barley aleurone cells, by specifically binding to a GA response element, the TAACAAA box, in the gene promoter and inducing gene expression (Gubler and Jacobsen 1992).

Interestingly, all five homeobox genes were down-regulated by GA. None of them showed more than a 1.4-fold change in response to ABA. Homeobox genes are known to be involved in the operation of differential genetic programs along the anterior-posterior axis of animal bodies (Gehring 1992). In plants, expression of the *KNOTTED1*-like homeobox (*KNOX*) genes in the shoot apical meristem are required for the maintenance of a functional meristem (Lincoln et al. 1994; Hay et al. 2002). Misexpression of the gene in *Arabidopsis*, including amount and timing, affects leaf morphology. Gibberellin activity suppresses the *KNOX* misexpression phenotype in *Arabidopsis*. The *KNOX* misexpression phenotype represses *AtGA20ox1* expression (Hay et al. 2003). Suppression of so many homeobox genes in barley aleurone tissues by GA may reveal a conserved part of GA signaling, which is worth further investigation. In addition, we observed that 19 of 24 zinc finger protein genes were up-regulated by GA, whereas the other five were down-regulated by GA. The *WRKY*, *NAM*, *bHLH*, and *Aux/IAA* genes were also differentially regulated by GA.

Three transcriptional factor genes were differentially regulated by ABA (Figure 6). The *WRKY* (Contig4386_at) gene and senescence-associated protein 1 (SEN1; Contig15259_at) were up-regulated by ABA, but down-regulated by GA. The *WRKY* gene is expressed in cold- and drought-treated barley tissue (Mare et al. 2004), whereas *SEN1* is found to be expressed during leaf senescence and in response to ABA in *Arabidopsis* (Oh et al. 1996; Hanaoka et al. 2002). In addition, a salt-tolerance protein (*STO*) gene (Contig6358_at) was down-regulated

Table 2. Transcriptional factor genes differentially regulated by GA and/or ABA

Barley 1 probe set ID	Fold-change (GA)	Fold-change (ABA)	Function annotation
HA11J15u_s_at	–5.11	1.15	APETALA2/Ethylene-responsive element binding protein family
Contig4826_at	–3.44	–1.23	Argonaute
Contig8572_s_at	12.44	–1.01	ARR
Contig8986_at	–6.81	–1.32	AT-hook DNA-binding protein (AHP1)
Contig13493_at	–18.22	–1.14	AtSR Transcription Factor family
Contig5251_at	–6.35	–1.60	Aux/IAA family
Contig8115_s_at	4.05	1.11	Aux/IAA family
HV_CEb0024B09r2_s_at	3.97	1.39	Aux/IAA family
Contig15125_at	3.02	–1.25	Aux/IAA family
Contig20506_at	15.03	–1.05	bHLH, basic Helix-Loop-Helix family factor
Contig6159_at	3.44	–1.10	bHLH, basic Helix-Loop-Helix family factor
Contig15975_at	18.91	–1.61	bHLH, basic Helix-Loop-Helix family factor
Contig8163_at	–4.53	–1.21	bZIP transcription factor family
Contig20055_at	–3.70	–1.45	DNA-binding protein, putative
Contig3395_at	4.66	–1.37	DNA-binding protein, putative
Contig4395_at	–3.62	1.17	EIN3-like(EIL) transcription factor family
HVSMEa0017I09r2_s_at	–4.53	1.08	EIN3-like(EIL) transcription factor family
Contig4741_s_at	–3.06	1.44	HB,homeobox transcription factor family
Contig6168_at	–7.26	–1.24	HB,homeobox transcription factor family
HVSMEn0016F09r2_s_at	–5.93	–1.08	HB,homeobox transcription factor family
Contig20612_at	–3.78	–1.07	HB,homeobox transcription factor family
Contig12869_at	–3.06	–1.25	Zinc finger homeobox family protein
Contig15595_at	–12.12	1.48	Heat-shock transcription factor family
Contig15230_at	5.77	1.63	Myb family transcription factor
X70876_at	5.15	–1.39	Myb family transcription factor
Contig14220_at	9.49	–1.00	Myb family transcription factor
Contig10555_at	3.56	–1.23	Myb family transcription factor
Contig13879_at	–13.02	–1.15	Myb family transcription factor
X87690_s_at	4.38	–1.26	Myb family transcription factor
HS18K19u_s_at	4.75	–1.10	Myb family transcription factor
Contig15670_at	3.59	–1.03	MYB-related transcription factor family
Contig13658_at	11.05	–1.52	No apical meristem (NAM) protein
Contig14026_at	13.46	–1.47	No apical meristem (NAM) protein
Contig6484_at	10.98	1.04	No apical meristem (NAM) protein
Contig6233_at	–3.83	1.19	No apical meristem (NAM) protein
Contig6233_s_at	–3.16	1.15	No apical meristem (NAM) protein
Contig6235_s_at	–3.51	1.70	No apical meristem (NAM) protein
Contig9031_at	6.60	–1.61	No apical meristem (NAM) protein
Contig14342_at	28.21	1.02	Nucleoid DNA-binding protein cnd41 - like protein
Contig9418_at	–3.02	–1.44	Nucleoid DNA-binding protein cnd41 - like protein
Contig4861_at	–4.66	–1.07	Remorin family protein
Contig15259_at	–5.99	4.13	Senescence-associated protein (SEN1)
Contig14754_at	–5.87	1.48	Transcriptional Adaptor Zinc Bundle domain family
Contig8519_at	17.58	–2.58	Trihelix, Triple-Helix transcription factor family
Contig6278_at	5.41	1.16	Trihelix, Triple-Helix transcription factor family
Contig4386_at	–7.34	3.57	WRKY family transcription factor
Contig10402_at	4.46	1.55	Zinc finger (C3HC4-type RING finger) family protein
Contig9457_at	5.56	–1.19	Zinc finger (C3HC4-type RING finger) family protein
Contig20287_at	–96.29	–1.32	Zinc finger (C3HC4-type RING finger) family protein
Contig11443_at	13.10	1.16	Zinc finger (C3HC4-type RING finger) family protein

Table 2 (continued).

Barley 1 probe set ID	Fold-change (GA)	Fold-change (ABA)	Function annotation
HVSMEg0010A16r2_s_at	10.02	1.11	Zinc finger (C3HC4-type RING finger) family protein
Contig14964_at	–4.48	1.01	Zinc finger (C3HC4-type RING finger) family protein
Contig10401_s_at	4.73	1.52	Zinc finger (C3HC4-type RING finger) family protein
Contig14351_at	–6.31	–1.08	Zinc finger (C3HC4-type RING finger) family protein
Contig2830_at	3.23	–1.48	Zinc finger (C3HC4-type RING finger) family protein
Contig24933_at	34.74	–1.14	Zinc finger (C3HC4-type RING finger) family protein
Contig14866_at	–3.18	1.22	Zinc finger (C3HC4-type RING finger) family protein
Contig17769_at	4.79	1.25	Zinc finger (C3HC4-type RING finger) family protein
Contig8204_at	3.69	1.16	Zinc finger (C3HC4-type RING finger) family protein
Contig11867_s_at	4.27	–1.38	Zinc finger (C3HC4-type RING finger) family protein
Contig4580_at	6.17	1.73	Zinc finger (C3HC4-type RING finger) family protein
Contig6585_at	3.35	–1.14	Zinc finger (CCCH-type) family protein(C3H)
Contig7881_at	5.12	2.10	Zinc finger (CCCH-type) family protein(C3H)
Contig5214_at	6.17	–1.89	Zinc finger (CCCH-type) family protein(C3H)
Contig15377_at	7.86	–1.95	C2C2(Zn) DOF zinc finger family
Contig9071_at	28.66	–1.92	C2C2(Zn) DOF zinc finger family
Contig13717_at	4.08	–1.20	C2C2(Zn) DOF zinc finger family
Contig17684_at	3.58	1.10	Zinc finger (GATA type) family protein
Contig23823_at	32.90	1.10	C2H2 zinc finger family
Contig6358_at	–3.33	–3.42	STO protein,constans-like zinc finger family

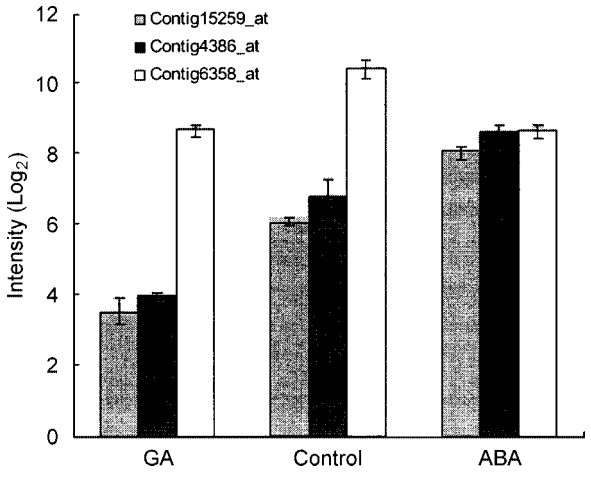

Figure 6. Expression of abscisic acid (ABA)-differentially regulated transcriptional factor genes.

Three transcriptional factor genes were differentially regulated by ABA: *WRKY* (Contig4386_at), a senescence-associated protein 1 (SEN1; Contig15259_at), and a salt-tolerance protein (*STO*) gene (Contig6358_at). The average intensity of each probe set and the intensity range of its three replicates are indicated on the *y*-axis.

by both ABA and GA. Its homolog in *Arabidopsis* has been shown to increase salt tolerance in yeast deficient in calcineurin (Lippuner et al. 1996). Overexpression of the *STO* in transgenic *Arabidopsis* plants results in higher salt tolerance (Nagaoka and Takano 2003).

Differential regulation of GA and ABA signal transduction pathways

Previous studies have shown that several events are likely to be involved in regulating GA and ABA responses in cereal aleurone in addition to transcriptional regulation, such as G-protein, calcium/calmodulin, protein phosphorylation cascade, and ubiquitin/26S proteasome-dependent protein degradation (reviewed by Lovegrove and Hooley 2000). Even though some genes are clearly known to be involved in the signal pathways, most have not been isolated in barley aleurone. Here, we summarize the transcriptional regulation of the genes potentially involved in GA and ABA signaling from our microarray data. Table 3 lists the GA- and/or ABA-responsive genes encoding G-proteins, calcium regulation proteins, protein kinases, or phosphatases.

GTP-binding proteins

A compound, Mas7, which stimulates GDP/GTP exchange by heterotrimeric G protein, can specifically induce α-amylase gene expression in wild oat aleurone protoplasts (Jones et al. 1998). In rice, it has been shown that the *d1* dwarf mutant is caused by mutation of the α-subunit of heterotrimeric G proteins

(Ashikari et al. 1999). In *Arabidopsis*, the G_α mutation, GPA1, affects seed germination (Ullah et al. 2002). We did not observe changes in expression of the homologous G_α protein gene (Contig19292_at). However, four GTP-binding protein genes were regulated by GA treatment (Table 3). Three putative Rho GTPase genes (Contig12082_at, HVSMEh-0084N15r2_s_at, and Contig25552_at) were up-regulated 17- to 40-fold by GA, whereas two of them were down-regulated 2.5- and threefold by ABA. In addition, a Ras-related protein (Contig10901_at) was down-regulated threefold by GA. The

Table 3. GA and/or ABA differentially regulated G-protein, calcium regulation, protein kinase or phosphotase genes

Barley 1 probe set ID	Fold-change (GA)	Fold-change (ABA)	Function annotation
Contig12082_at	27.77	–2.49	Rho GTPase
HVSMEh0084N15r2_s_at	17.04	–3.24	Rho GTPase
Contig25552_at	40.15	–1.08	Rac-like GTP-binding protein
Contig10901_at	–3.37	1.20	Ras-related protein
Contig7339_s_at	3.54	–1.46	Calmodulin-binding protein
HA10M05u_s_at	8.12	–1.08	Calmodulin-binding protein
Contig13493_at	–18.22	–1.14	Calmodulin-binding protein
Contig8468_at	–5.93	2.27	Calmodulin-binding protein
Contig11952_at	3.13	1.32	Calmodulin-binding protein
Contig15997_at	19.75	–1.72	Calcium-dependent protein kinase
Contig6447_at	–4.21	–1.15	Calcium-dependent protein kinase
Contig6268_at	4.61	–1.08	C2 domain-containing protein
Contig24167_at	-6.48	1.14	C2 domain-containing protein
Contig24962_at	4.75	1.36	Calcineurin B-like protein 1
Contig1560_at	3.18	1.11	Calnexin 1
Contig7147_at	11.05	1.14	Calcium-binding EF hand family protein
Contig14555_s_at	7.47	1.08	Polcalcin Jun o 2
Contig8829_at	6.83	–1.31	Polcalcin Jun o 3
Contig1903_at	7.60	–1.28	Calreticulin 1
rbags16g09_s_at	6.22	–1.19	Calreticulin 1
HV_CEb0006A14f_s_at	1.16	7.82	Touch-responsive protein
Contig15719_at	5.41	–1.17	CBL-interacting protein kinase
Contig15820_at	29.19	–1.02	CBL-interacting protein kinase
Contig4152_at	–5.29	–1.04	CBL-interacting protein kinase
Contig14415_at	–4.09	2.82	Protein kinase
Contig14879_at	–12.68	–1.73	Protein kinase
Contig16082_at	3.71	–1.34	Protein kinase
Contig9077_at	5.04	1.08	Protein kinase
Contig19616_at	5.01	–1.18	Protein kinase
Contig7326_at	3.98	1.26	Protein kinase
Contig7505_at	–3.36	1.21	Protein kinase
Contig9035_at	–4.12	1.03	Protein kinase
Contig13334_at	–3.92	–1.27	Leucine-rich repeat transmembrane protein kinase
Contig7672_at	–6.49	3.78	Protein phosphatase 2C (PP2C)
Contig13811_at	–21.54	3.84	Protein phosphatase 2C (PP2C)
Contig11409_at	–4.41	1.41	Protein phosphatase 2C (PP2C)
Contig20314_at	3.90	–1.14	Protein phosphatase 2C (PP2C)
Contig10323_at	–7.47	–1.27	Protein phosphatase 2C (PP2C)
Contig11720_at	3.42	1.75	Protein phosphatase 2C (PP2C)
HS01M21w_s_at	3.23	1.63	Protein phosphatase 2C (PP2C)
Contig13376_at	–3.09	1.65	Protein phosphatase 2C (PP2C)
Contig19338_at	5.58	–1.67	Tyrosine phosphatase-like
Contig7617_at	17.51	1.04	Tyrosine phosphatase-like

differential regulation is consistent with previous results that GTP-binding proteins are involved in GA and ABA signaling in cereal aleurone (Homann and Tester 1997; McCubbin et al. 2004).

Calcium regulation proteins

It has been suggested that the calcium signal is involved in the GA and ABA response in barley aleurone cells (Gilroy 1996; Ritchie and Gilroy 1998). Some GTP-binding activity and kinase activity in the activated aleurone are dependent on calcium (Homann and Tester 1997; McCubbin et al. 2004). In the present study, we observed that many calcium-related genes were differentially regulated by GA, including five calmodulin-binding family proteins, two calcium-dependent protein kinases, and nine other calcium-binding or related proteins (Table 3). Therefore, it is highly possible that calcium and calmodulin are involved in the GA response in aleurone. We also observed that a calmodulin-related protein gene (HV_CEb0006A14f_s_at) was up-regulated eightfold by ABA (Table 3).

Ubiquitin/26S proteasome-mediated protein degradation

Recent evidence has shown that ubiquitin/26S proteasome-mediated protein degradation plays an important role in regulating GA signal pathways in plants (Sasaki et al. 2003). F-box proteins are believed to function as a major determination factor for the specificity with which the SKP1/Cullin/F-box protein complex (SCF) selects the target protein for degradation (Vierstra 2003). Increasing evidence shows that, in response to GA, the F-box proteins act on the DELLA proteins (RGA in *Arabidopsis*, SLN1 in barley, and SLR1 in rice), the negative regulator in GA signal pathways (McGinnis et al. 2003; Sasaki et al. 2003; Dill et al. 2004; Gomi et al. 2004). α-Amylase could not be induced by GA in de-embryonated half-seeds of the F-box mutant *gid2* rice (Sasaki et al. 2003). We observed that nine putative F-box proteins were up-regulated by GA (Figure 7). Although no F-box gene has been identified to be involved in GA signal pathway in barley so far, the DELLA protein, SLN1, was found to rapidly disappear with GA treatment and proteasome inhibitor blocked this response (Chandler et al. 2002; Fu et al. 2002; Gubler et al. 2002). A dynamic interaction between SLN1 protein and GA content was found to be involved in controlling leaf elongation rate in barley (Chandler et al. 2002). We also observed that one putative ubiquitin-activating E1 protein gene (Contig15306_at), one RING/U-box E3 gene (Contig15752_s_at), and one ubiquitin protease gene (Contig6229_s_at) were up-regulated by GA (Figure 7). These observations suggest that the ubiquitin/26S proteasome pathway is likely to be activated in the GA-treated aleurone. Interestingly, among the nine putative F-box genes, Contig12407_at is down-regulated more than threefold by ABA. This result raises the possibility that ABA may counteract GA

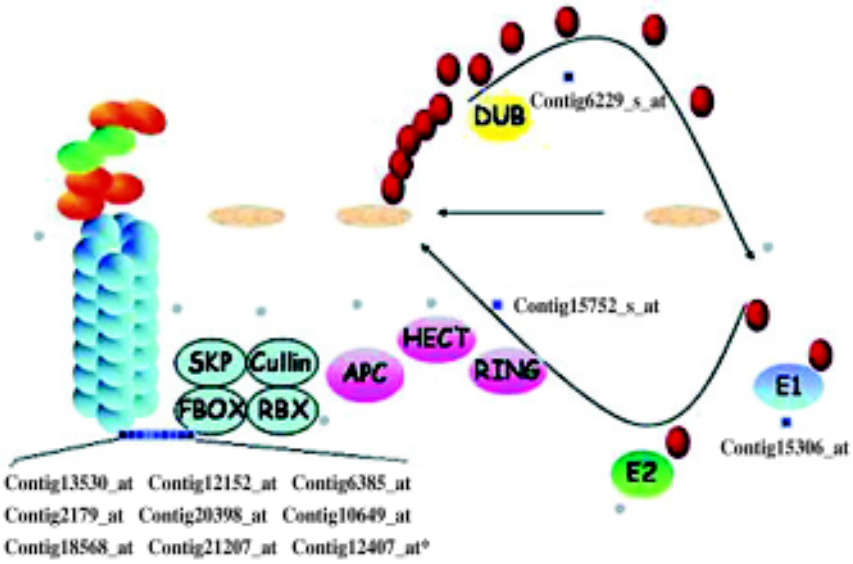

Figure 7. Giberrellin (GA)-differentially regulated genes in the ubiquitin/26S proteasome pathway.

The diagram of the ubiquitin/26S proteasome pathway is shown based on MapMan (Thimm et al. 2004). The blue square represents the genes up-regulated by GA. Nine probe sets representing F-box proteins were up-regulated by GA and are marked. The Contig15306_at encoding ubiquitin-activating E1 protein, Contig6229_s_at encoding ubiquitin protease, and Contig15752_s_at encoding RING/U-box E3 protein were up-regulated by GA and are marked in the pathway.

by inhibiting ubiquitin protein degradation. Further characterization of these genes may lead to a clearer understanding of ubiquitin/26S proteasome degradation in the GA and ABA signaling pathways.

Protein phosphorylation/de-phosphorylation

Protein phosphorylation/de-phosphorylation has regulatory functions in GA and ABA signaling pathways (Kuo et al. 1996; Ritchie and Gilroy 1998). Several protein kinase and phosphatase genes have been identified in cereal aleurone (Gomez-Cadenas et al. 1999; McCubbin et al. 2004). We observed that 12 protein kinase genes were differentially regulated by GA. Six were up-regulated, whereas six were down-regulated (Table 3). We did not observe any kinase gene that was differentially regulated more than threefold by ABA. However, we observed that one kinase gene (Contig14415_at) was down-regulated 12.6-fold by GA and up-regulated 2.8-fold by ABA. The *HvPKABA1* (Contig7433_at), which has been shown to be involved in GA and ABA signaling in aleurone (Gomez-Cadenas et al. 1999, 2001), was up-regulated 2.8-fold by ABA and down-regulated 1.5-fold by GA.

Of 12 GA-responsive kinase genes, three encoded putative calcineurin B-like (CBL)-interacting protein kinases (CIPK). Two (Contig15719_at and Contig15820_at) were up-regulated by five- and 29-fold, respectively, whereas one (Contig4152_at) was down-regulated by fivefold. The CIPK is a group of Ser/Thr protein kinases that specifically interact with CBL proteins, calcium sensors. The CBL proteins are believed to recognize specific calcium signals and relay these signals into downstream response such as phosphorylation cascades and regulation of gene expression (Kim et al. 2000). Interestingly, we also observed that one *CBL* gene (Contig24962_at) was up-regulated fivefold following GA treatment (Table 3). In addition, a putative Shaggy-related protein kinase gene (Contig9035_at) was down-regulated fourfold by GA. Expression of its *Arabidopsis* homolog gene, *AtGSK*, can rescue a yeast calcineurin mutant (Piao et al. 1999). All the data raise the possibility that CBL/CIPK calcium signals may be involved in the GA response pathway. Recent research has shown that CBL/CIPK calcium signaling is involved in the ABA response in *Arabidopsis* (Kim et al. 2003; Pandey et al. 2004). The *CIPK3* and *AtGSK1* genes are inducible by ABA and expression of ABA-responsive genes is reduced in the mutant, *cipk3*, in *Arabidopsis* (Piao et al. 1999; Kim et al. 2003). However, none of the barley homologous *CIPK* or Shaggy-like genes were significantly regulated by ABA in our microarray data. Instead, they were down-regulated by GA.

We also observed that five protein phosphatase genes were up-regulated by GA and five were down-regulated (Table 3). Among these genes, two Protein Phosphatase 2C (PP2C) genes (contig7672_at and Contig13811_at) were down-regulated six- and 20-fold by GA, respectively, but up-regulated three- and fourfold by ABA, respectively. It has been well documented that PP2Cs are involved in regulating the ABA response in plant cells. In *Arabidopsis,* Abscisic Acid-Insensitive (ABI) 1 and ABI 2 play negative regulatory functions in the ABA signal pathway (Leung and Giraudat 1998). Expression of *Arabidopsis ABI1* in barley aleurone blocked expression of ABA-induced genes (Shen et al. 2001). Recently, *AtP2C-HA* was found to be highly up-regulated by ABA in guard cells and the loss-of-function mutant conferred ABA-hypersensitive regulation of stomatal closing and seed germination in *Arabidopsis* (Leonhardt et al. 2004).

Potential involvement of other signal pathways in the GA and ABA response

We observed that a number of genes potentially involved in phytohormone biosynthesis, degradation, and signal transduction were differentially regulated by GA or ABA. These included GA, ABA, cytokinin, jasmonates, brassinosteroids, ethylene, and auxin. These data suggest that GA and ABA are involved in a sophisticated and complex signaling network in barley aleurone cells.

We observed that a putative gibberellin 20-oxidase (Contig7693_at) was down-regulated 3.1-fold by GA (Figure 8). Gibberellin 20-oxidase catalyses several steps in the GA biosynthetic pathway to produce bioactive GA_1 and GA_4 (Hedden and Proebsting 1999), and is subject to GA feedback regulation in *Arabidopsis* and potato (*Solanum tuberosum*; Phillips et al. 1995; Carrera et al. 1999). During *Arabidopsis* seed germination, GA20-oxidase genes are induced transiently at the early stage. The GA20-oxidase gene was believed to be the target in GA feedback regulation (Ogawa et al. 2003). Although there is no evidence that *de nevo* GA biosynthesis occurs in aleurone tissues during seed germination, it is likely that the conserved feed-back regulation of the GA biosynthetic pathway is preserved in barley aleurone and is activated when treated with exogenous GA. As a key enzyme, GA 20-oxidase is also subject to light regulation in potato (Jackson et al. 2000) and brassinosteroid regulation in *Arabidopsis* (Bouquin et al. 2001).

We also observed that two GA-responsive genes were potentially involved in the cytokinin degradation and signaling pathway (Figure 8). The Contig16024 encoding a cytokinin oxidase, which participated in cytokinin degradation (Armstrong 1994; Schmulling et al. 2003), was up-regulated 27-fold; the Contig8572_s_at, a homolog of *ARR6*, was up-regulated 12-fold. ARR6 in *Arabidopsis* mediates a negative feedback response in the cytokinin signaling pathway (Hwang and Sheen 2001).

An allene oxide synthase (*AOS*) gene (Contig3097_at), a member of the cytochrome p450 CYP74 gene family, was up-regulated threefold by ABA, but down-regulated 10-fold by GA

(Figure 8). This enzyme catalyses dehydration of the hydroperoxide to an allene oxide, the first step in the biosynthesis of jasmonates (Song et al. 1993; Laudert and Weiler 1998). In *Arabidopsis*, the loss-of-function mutation of the *CYP74* gene blocks jasmonic acid biosynthesis and has a phenotype of male sterility and defective wound signal transduction (Park et al. 2002). The changes in expression of the *CYP74* gene may raise the possibility that biosynthesis of jasmonate is subject to GA and ABA antagonistic regulation in barley aleurone cells.

It is likely that the brassinosteroid biosynthetic pathway was regulated by GA. The Contig5362_at, a homolog of *DIM/DWF1* in *Arabidopsis*, was down-regulated sixfold by GA, but was not affected by ABA (Figure 8). This gene is responsible for conversion of 24-methylenecholesterol to campesterol in the brassinosteroid biosynthetic pathway (Choe et al. 1999). Its homologs in maize and rice have also been identified recently (Tao et al. 2004; Hong et al. 2005). Loss of function of the gene results in dwarfism (Azpiroz et al. 1998) and affects the expression of the β-tubulin gene *TUB1*, which is thought to be important for plant cell growth (Takahashi et al. 1995).

Eleven GA down-regulated genes were homologous to genes previously identified as ABA-related genes in plant species (Table 4). Eight of the 11 probe sets were shown to be up-regulated by ABA. Six of the probe sets represent the genes homologous to *AtEm6* and two probe sets are homologous to *AtEm1* in *Arabidopsis*. Both *AtEm6* and *AtEm1* encode late embryogenesis abundant (LEA) protein and are positively regulated by ABI3 and ABI5 in *Arabidopsis* (Gaubier et al. 1993). It has been shown that ABA leads to a post-transcriptional accumulation and activation of ABI3, and ABI3 then activates ABI5 activity, which directly binds to promoter of *AtEm6* (Lopez-Molina et al. 2001, 2002; Nakamura et al. 2001). We observed that the expression of barley *ABI3* (Contig10484_at) and *ABI5* (Contig8163_at) genes were down-regulated by GA, but unchanged by ABA. A recent report has demonstrated that *HvABI5* is subject to autoregulation in barley aleurone (Casaretto and Ho 2005). HvABI5 and ABI3 (HvVP1) are necessary for the ABA-induced gene expression, but have no effect on the GA-induced and ABA-suppressed gene expression, such as α-amylase (Casaretto and Ho 2003, 2005). In addition, the gene encoding a putative aldehyde oxidase 3 (Contig4917_at), which catalyses the last step in the ABA biosynthetic pathway (Seo et al. 2000), was down-regulated 26-fold by GA. This result could suggest that there may be some ABA biosynthetic activities in barley aleurone (Seo et al. 2004), but they may be completely blocked by GA treatment.

We observed that 14 genes differentially regulated by GA were related to the ethylene biosynthetic and signaling pathways (Table 4), of which six were up-regulated and eight were down-regulated. The *AtERF5* and *AtERF4* genes encode ethylene-responsive element binding factors in *Arabidopsis*. In response to ethylene, *AtERF5* functions as a positive transcriptional regulator, whereas *AtERF4* functions as a negative transcriptional regulator (Fujimoto et al. 2000). The *AtERF5*-like gene (Contig17873_at) was up-regulated threefold by GA and the *AtERF4*-like gene (Contig7722_at) was down-regulated 22-fold by GA but up-regulated twofold by ABA. In addition, an *ETR1* (ethylene receptor 1) gene (Contig4907_s_at) was up regulated 19-fold by GA, whereas two *EIL1* (ethylene-insensitive3-like 1) genes (Contig4395_at and HVSMEa0017I09r2_s_at) were down-regulated threefold by GA. The *ETR1* gene encodes a histidine kinase ethylene receptor; *EIL1* encodes an ethylene responsive transcription factor. Both these genes are involved in the ethylene signaling pathway in *Arabidopsis* (Chao et al. 1997; Zhao et al. 2002). In addition, three probe sets (Contig10361_at, Contig9486_at, and HVSMEi0013O11r2_at) represent ethylene-forming enzyme-like genes and were down-regulated 10-, five- and 25- fold by GA, respectively. Many genes related to ethylene were differentially regulated by GA and ABA, suggesting that they interact

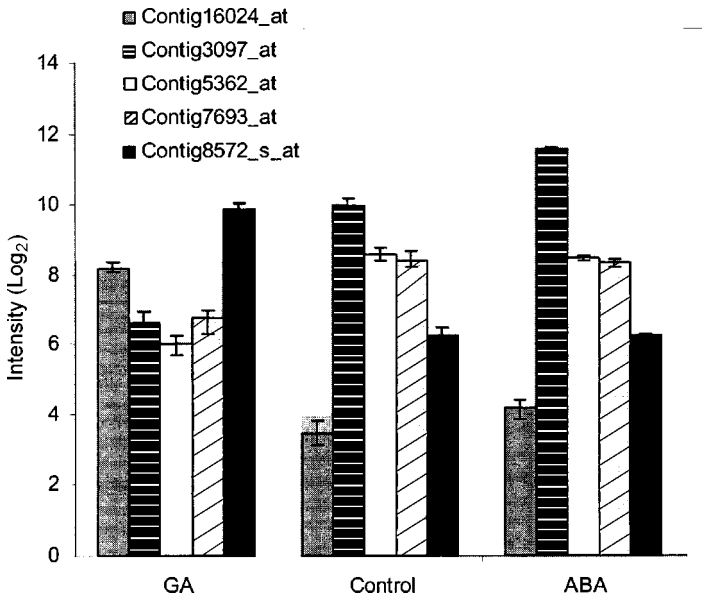

Figure 8. Differentially regulation of the genes in giberrellin (GA), brassinosteroid, cytokinin and jasmonate metabolic and signaling pathways.

Contig7693_at, gibberellin 20-oxidase; Contig16024_at, cytokinin oxidase; Contig8572_s_at, two-component responsive regulator/response regulator 6 (ARR6); Contig5362_at, *Dwarf1* in *Arabidopsis*, cell elongation protein; Contig3097_at, allene oxide synthase. The average intensity of each probe set and the intensity range of its three replicates are indicated on the *y*-axis.

strongly with each other.

Gibberellin and ABA differentially regulated a large group of genes related to auxin (Table 4). Contig5433_at, a homolog to auxin-induced protein ATB2 in *Arabidopsis*, was up-regulated threefold by ABA and 2.7-fold by GA. However, another gene (Contig3852_at), which belongs to the same family, was down-regulated sixfold by GA. The Contig8128_at, a homolog of the *Arabidopsis* auxin influx carrier AUX1 (Bennett et al. 1996), was up-regulated more than 20-fold by GA. Of three GA responsive genes, namely Contig5580_s_at, Contig5581_at, and rbaal12n12_s_at, which are homologous to an *Arabidopsis* auxin efflux carrier domain gene, two were up-regulated 85- and 88-fold by GA, whereas one was down-regulated threefold by GA. In addition, we also observed that four probe sets homologous to *Auxin/IAA* genes in *Arabidopsis* (Liscum and Reed 2002), namely Contig15125_at, Contig8115_s_at,

Table 4. Differentially regulated ABA-, ethylene- or auxin-related genes

Barley 1 probe set ID	Fold-change (GA)	Fold-change (ABA)	Function annotation
Contig1830_at	–3.61	3.45	ABA-regulated gene (AtEM6)
Contig1834_at	–25.59	4.46	ABA-regulated gene (AtEM6)
Contig1832_x_at	–7.13	10.66	ABA-regulated gene (AtEM6)
Contig1832_s_at	–10.52	13.53	ABA-regulated gene (AtEM6)
Contig1832_at	–9.24	9.20	ABA-regulated gene (AtEM6)
Contig1830_at	–3.62	3.44	ABA-regulated gene (AtEM6)
Contig1838_at	–10.64	3.81	ABA-regulated gene (AtEM1)
Contig1839_at	–7.19	5.61	ABA-regulated gene (AtEM1)
Contig8163_at	–4.53	–1.21	Abscisic acid insensitive 5 (ABI5)
Contig10484_at	–3.77	–1.00	Abscisic acid-insensitive protein 3 (ABI3)
Contig4917_at	–26.64	–1.28	Aldehyde oxidase 3 (AAO3)
Contig3321_at	–3.40	1.29	Ethylene-responsive ER6 protein
Contig3532_at	3.39	–1.02	Hypothetical protein ER6 protein
Contig3532_s_at	3.01	1.13	Hypothetical protein ER6 protein
Contig7507_at	–16.78	1.05	Hypothetical protein ER6 protein
Contig10361_at	–10.06	1.94	Ethylene-forming-enzyme-like dioxygenase-like protein
Contig9486_at	–4.35	1.41	Ethylene-forming-enzyme-like dioxygenase-like protein
HVSMEi0013O11r2_at	–22.93	1.12	Ethylene-forming-enzyme-like dioxygenase-like protein
Contig4907_s_at	19.48	–1.01	Ethylene-response protein, ETR1
Contig4395_at	–3.62	1.17	Ethylene-insensitive3-like1 (EIL1)
HVSMEa0017I09r2_s_at	–4.53	1.08	Ethylene-insensitive3-like1 (EIL1)
Contig658_s_at	5.35	1.45	Ethylene-responsive DEAD box RNA helicase
Contig7722_at	–22.26	2.28	Ethylene responsive element binding factor 4 (AtERF4)
Contig17873_at	3.20	1.25	Ethylene responsive element binding factor 5 (ATERF5)
Contig18796_at	9.37	1.30	Ethylene-responsive transcriptional coactivator
Contig5433_at	2.68	3.24	Auxin-induced protein(atb2)
Contig3852_at	–3.01	–1.27	Putative similar to auxin-induced atb2
Contig8128_at	23.56	–1.00	Amino acid permease, putative (AUX1)
rbaal12n12_s_at	–3.78	1.26	Auxin efflux carrier family protein
Contig5580_s_at	86.88	–1.23	Auxin efflux carrier family protein
Contig5581_at	85.55	–1.14	Auxin efflux carrier family protein
Contig5251_at	–6.35	–1.60	AUX/IAA family protein
Contig15125_at	3.02	–1.25	Auxin regulated protein (IAA13)
Contig8115_s_at	4.05	1.11	Auxin-responsive protein (IAA18)
HV_CEb0024B09r2_s_at	3.97	1.39	Auxin-responsive protein (IAA18)
Contig12102_at	5.56	–1.34	Auxin-responsive GH3 family protein
Contig9624_at	–15.38	–1.20	Auxin down-regulated protein ARG10
Contig1762_s_at	–5.61	1.23	Auxin-regulated protein
Contig9093_at	32.63	–1.48	Auxin-induced protein AIR12
Contig9591_at	11.34	1.11	Auxin-induced protein AIR13

HV_CEb0024B09r2_s_at, and Contig5251_at, were differentially regulated by GA.

Genes differentially regulated by both GA and ABA

Among the 1 328 GA-responsive genes and the 206 ABA-responsive genes, 83 were differentially regulated by both GA and ABA (Table 5). Forty percent of the ABA-responsive genes were also differentially regulated by GA. Sixty-six percent of the ABA-down-regulated genes were also differentially regulated by GA. Thus, the GA- and ABA-responsive genes overlapped significantly, and majority of ABA-down-regulated genes were also differentially regulated by GA. The genes differentially regulated by GA and ABA could be assigned into four groups based on their expression in response to GA and ABA. They were either up-regulated by both GA and ABA (up-up gene), up-regulated by GA but down-regulated by ABA (up-down gene), down-regulated by both GA and ABA (down-down gene), or down-regulated by GA but up-regulated by ABA (down-up gene).

Interestingly, we observed that a significant percentage of genes differentially regulated by both GA and ABA (27%) showed a coordinated response to GA and ABA (up-up or down-down). Nine genes were up-regulated by GA and ABA and encoded cytochrome P450, dehydrin, glucanase, lipase, hemoglobin apoprotein, inorganic pyrophosphatase, and nicotianamine aminotransferase. Thirteen genes were down-regulated by both GA and ABA. Contig6358_at was the salt tolerance (STO) protein gene discussed earlier. Some of the genes, such as a metallothionein-like protein gene, a heat shock protein gene, UVB-resistance genes, and a hydroxyproline-rich glycoprotein gene, are stress-related genes. A zinc finger protein gene and an α-amylase inhibitor gene were also down-regulated by both ABA and GA. To our knowledge, few genes have been identified so far to be coordinately regulated by both GA and ABA (up-up and down-down).

Of the 83 genes, 73% of the genes (up-down and down-up genes) were subject to antagonistic regulation by ABA and GA. Twenty-six genes were up-regulated by GA but down-regulated by ABA. Most of the genes in this group encoded hydrolases and were likely to participate in storage mobilization during seed germination, as described above. For example, two α-amylase genes and four cysteine protease genes were included in this group. In addition, two probe sets (Contig12147_at and HVSMEb0012C16r2_s_at) representing one putative purple acid phosphatase gene were induced more than 66-fold by GA and suppressed more than threefold by ABA. Because purple acid phosphatase was identified to have phytase activity in soybean (Hegeman and Grabau 2001), these genes may be involved in the degradation of phytin inclusions in aleurone to release phosphate for embryo growth and seedling development. Up-regulation of a phosphate-responsive protein-like gene (Contig12147_at) supports that the release of phosphate was induced by GA from barley aleurone. We also observed that 35 genes were induced by ABA, but suppressed by GA. A large proportion of the genes encoded seed storage and desiccation tolerance-related genes, such as *LEA*, *Em*, and *dehydrin*. Ten probe sets representing *LEA* and one *Em* gene were induced by ABA, but suppressed by GA. The expressions of these genes have been shown to be induced during seed maturation and desiccation processes as ABA levels increase and GA levels decrease (Bewley and Black 1994; White et al. 2000). In addition, the genes encoding osmotin-like protein, cinnamyl alcohol dehydrogenease, RAFTIN1a anther proteins, and several other genes were up-regulated by GA and down-regulated by ABA. The WRKY transcription factor (Contig4386_at), PP2C protein phosphotases (Contig7672_at, Contig13811_at), AP2-domain DNA-binding protein (Contig24555_at), DNA-binding protein Dof2 (Contig19502_at), allene oxide synthase (Contig3097_at), and senescence-associated protein (Contig15259_at) were also found in this up-down list. The potential regulatory functions of the genes may raise the possibility that the genes may play roles in regulating the antagonistic response to GA and ABA.

Many antagonistically regulated genes encode seed maturation and germination-related proteins, further supporting that ABA and GA play antagonistic roles in regulating seed maturation and germination (Bewley and Black 1982). During seed maturation, the ABA content increases markedly, but the GA content is very low in most plant species (King 1976; Finkelstein et al. 1985; Black 1991). It is believed that ABA induces the production of seed storage proteins and desiccation-tolerant proteins to prepare the seeds to go through desiccation stages and provide a reserve for later seed germination. Abscisic acid also suppresses gene expression involved in seed germination to prevent vivipary, such as hydrolytic enzyme genes (Hoecker et al. 1999). In contrast, the GA content increases markedly, whereas the ABA content drops significantly during seed germination and seedling growth (Jacobsen 1995). The GA and ABA content switch is likely to lead to a seed developmental switch from maturation and dormancy stages to germination and seedling growth stages. Meanwhile, a high level of GA prevents the synthesis of storage proteins and desiccation-related proteins, which may consume the nutrient and energy resources used in germination and seedling growth. Such a GA and ABA antagonistic regulation of gene expression during seed maturation and germination, which evolved in plants, provides an effective and elegant way to control the developmental switch from seed maturation and germination.

Table 5. The genes differntially regulated by both GA and ABA

Barley 1 probe set ID	Fold-change (GA)	Fold-change (ABA)	Function annotation
Contig11708_at	3.69	4.66	Cytochrome P450
Contig15561_s_at	3.60	7.89	Cytochrome P450
Contig1709_at	3.87	3.15	Dehydrin 7
Contig20715_at	7.58	4.86	Glucanase
Contig15214_at	8.43	24.81	Lipase-like protein
Contig13656_at	4.64	4.27	Unknow
Contig3995_s_at	6.08	5.46	Haemoglobin apoprotein
Contig2021_at	5.26	3.59	Inorganic pyrophosphatase
Contig7287_at	113.06	3.99	Nicotianamine aminotransferase
Contig1383_s_at	–4.43	–4.51	Metallothionein-like protein type 3
Contig6358_at	–3.33	–3.42	Zinc finger protein
Contig49_x_at	–3.05	–3.08	Alpha-amylase inhibitor
Contig12460_at	–4.25	–3.81	NBS-LRR disease resistance protein
Contig9758_s_at	–22.64	–3.26	Leucine-rich repeat family protein
HB18H23r_s_at	–3.04	–3.80	Heat shock protein
Contig25699_at	–97.33	–3.53	Integral membrane protein
Contig18021_at	–5.45	–3.73	Tubulin-tyrosine ligase family protein
HV09J08u_s_at	–5.96	–4.68	Globulin-2
Contig4218_at	–7.36	–3.50	Phosphatidylinositol 3- and 4-kinase family protein
Contig9981_at	–4.69	–3.93	Hydroxyanthranilate hydroxycinnamoyltransferase 2
Contig1246_at	–8.89	–7.26	Hydroxyproline-rich glycoprotein family protein
Contig9934_at	–8.20	–3.46	UVB-resistance protein
Contig7087_at	39.83	–3.06	Alpha-amylase type A
Contig7088_at	35.07	–5.38	Alpha-amylase type A
rbaal21f05_s_at	5.38	–7.49	Cysteine proteinase
Contig3901_s_at	9.04	–9.75	Cysteine proteinase
Contig5281_at	12.29	–4.54	Cysteine proteinase
Contig3900_at	7.86	–3.52	Cysteine proteinase
Contig16010_at	73.04	–16.22	Putative 1,4-beta-xylanase
Contig2834_at	14.40	–54.40	Beta-glucanase
Contig5703_at	15.24	–3.90	Beta-*D*-xylosidase
Contig13792_s_at	113.82	–7.23	1,4-Beta-*D*-xylan xylanohydrolase
HVSMEb0012C16r2_s_at	66.33	–6.40	Putative purple acid phosphatase
Contig4453_at	145.32	–3.14	Putative purple acid phosphatase
Contig12147_at	74.84	–4.63	Phosphate-responsive protein
Contig2964_at	13.80	–3.69	Fructose-1,6-bisphosphatase
rbah15o14_s_at	14.57	–4.57	Fructose-1,6-bisphosphatase
EBpi07_SQ002_J15_at	8.47	–4.37	Cinnamyl alcohol dehydrogenase
Contig8708_at	22.38	–5.60	Nodulin MtN3 family protein
Contig5067_at	4.97	–5.29	Nicotianamine synthase 9
HM09F12r_s_at	5.48	–11.98	RAFTIN1a anther protein
Contig19330_at	61.47	–12.24	Putative GDSL-motif lipase/hydrolase protein
Contig13013_at	8.16	–4.52	Putative polygalacturonase
Contig11326_at	3.15	–3.84	Putative acetyl-CoA carboxylase
Contig9094_at	4.17	–4.61	Osmotin-like protein
EBem09_SQ003_F16_s_at	16.79	–6.73	Unknown (*Arabidopsis thaliana*)
Contig12407_at	15.66	–3.20	Unknown (*Arabidopsis thaliana*)
Contig6804_at	19.23	–4.56	Unknown (*Arabidopsis thaliana*)
Contig2406_at	–3.43	6.35	LEA2 protein
EBro08_SQ007_B12_s_at	–3.89	5.39	LEA2 protein

Table 5 (continued).

Barley 1 probe set ID	Fold-change (GA)	Fold-change (ABA)	Function annotation
Contig1830_at	–3.62	3.44	LEA protein 19.1
Contig1830_s_at	–3.56	6.29	LEA protein 19.1
Contig1832_s_at	–10.52	13.53	LEA protein 19.1
Contig1832_at	–9.24	9.20	LEA protein 19.1
Contig1832_x_at	–7.13	10.66	LEA protein 19.1
Contig1838_at	–10.64	3.81	LEA protein 19.3
Contig1839_at	–7.19	5.61	LEA B19.4
Contig1834_at	–25.59	4.46	Embryonic abundant protein
Contig1721_at	–6.72	3.65	Dehydrin 12
Contig13811_at	–21.54	3.84	Protein phosphatase 2C
Contig7672_at	–6.49	3.78	Protein phosphatase 2C
Contig17447_at	–4.34	3.47	Putative pectin-glucuronyltransferase
HVSMEh0081I20r2_s_at	–4.22	5.83	Cinnamyl alcohol dehydrogenase
Contig13706_at	–3.17	3.58	Serine-rich protein
Contig19502_at	–4.19	3.39	DNA-binding protein Dof2
EBro07_SQ003_G24_s_at	–17.24	3.52	Glycine rich protein
rbags1d02_at	–3.41	3.80	Nodulin MtN3 family protein
Contig16327_at	–30.08	4.47	Little protein 1
Contig3097_at	–10.17	3.10	Allene oxide synthase
Contig7871_s_at	–3.87	3.54	Phosphatidylinositol transfer-like protein III
Contig14304_at	–5.89	3.05	Glutathione S-transferase GST 42
Contig5995_at	–4.30	3.51	Acidic endochitinase
Contig15259_at	–5.99	4.13	Senescence-associated protein
Contig7736_at	–9.48	4.18	Glyoxalase I family protein
Contig4386_at	–7.34	3.57	WRKY transcription factor
Contig3811_at	–41.72	3.39	Galactinol synthase
Contig24555_at	–10.51	3.95	AP2-domain DNA-binding protein
Contig18451_at	–15.13	6.40	Unknown
Contig6741_s_at	–5.82	5.97	Unknown
HF25I22r_at	–16.80	3.21	Unknown
Contig6741_at	–3.93	4.45	Unknown
Contig7003_at	–5.23	4.36	Unknown
Contig11450_at	–6.55	3.19	Unknown

Materials and Methods

Plant material and treatment

Barley grains (*Hordeum vulgare* L. cv Himalaya), harvested in 1998 (provided by the Department of Agronomy, Washington State University, Pullman, WA, USA) were used in the experiments. Embryos were removed to produce de-embryonated half-grains. The half-grains were surface sterilized and then imbibed in 10 mmol/L $CaCl_2$ for 3 d. Aleurone tissue was isolated by gently removing the starchy endosperm. The isolated aleurone tissues were incubated in 10 mmol/L $CaCl_2$ (control) or 10 mmol/L $CaCl_2$ containing 1 μmol/L GA_3 (GA treatment), 50 μmol/L ABA (ABA treatment), or a mixture of 1 μmol/L GA_3 and 50 μmol/L ABA (GA plus ABA treatment) in Petri dishes with continuous shaking for 15 h at 25 °C. Three independent biological replications were conducted for each treatment. Each replication represented an independent treatment. The treated tissues were harvested and washed with water and frozen immediately in liquid nitrogen and stored at –80 °C for α-amylase activity assay and RNA isolation.

α-Amylase assays

α-Amylase activity was measured using the DNSA assay (Skadsen 1993). Maltose (Sigma, St Louis, MO, USA) was used as a standard to calculate enzyme activity. The total quantity of

soluble proteins in the extraction was measured using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The resulting α-amylase activity is expressed as μmol maltose/μg protein per h.

RNA extraction

A phenol extraction method was modified to extract total RNA from treated tissues. Aleurones were ground in liquid nitrogen and the powder was extracted in a mixture of equal amounts of extraction buffer (4% *p*-aminosalicylic disodium, 1% 1,5-naphthalenedisulfonic acid) and phenol. After mixing well, the same volume of chloroform was added and separated by centrifugation at 10 000 r/min for 15 min at 4 °C. The supernatant was transferred to another tube. The RNA was precipitated with ethanol. The pellet was dissolved in water and RNA was re-precipitated with LiCl (Sambrook and Russell 2001). The RNA for microarray was further purified by using RNeasy kits (Qiagen, Hilden, Germany). The quality and quantity of the RNA were determined using Nano-Drop (Agilent Technologies, Palo Alto, CA, USA) and Agilent 2100 bioanalyzers (Agilent Technologies).

Probe labeling and hybridization to Barley 1 GeneChip

A 22K Barley1 GeneChip (Close et al. 2004) from Affymetrix (Santa Clara, CA, USA) was used in the present studies. Probe labeling and hybridization were conducted as described in the manual provided by Affymetrix. A 10 μg sample of RNA was used for cDNA synthesis. Double-stranded cDNA was purified and 5 μL cDNA was used to generate a biotinylated cRNA target. Labeled cRNA was purified. A 20 μg sample of cRNA, at a final concentration of 0.5 μg/μL, was used for fragmentation. Then, 15 μg fragmented cRNA per hybridization was used to make the cocktail and 10 μg of equivalents was hybridized to a GeneChip. Hybridization was performed in an Affymetrix hybridization oven (model 640). The chips were washed and stained with streptavidin-phycoerythrin in the Affymetrix GeneChip fluidics station (model 400) and stained chips were scanned immediately with an Agilent 2500A GeneArray scanner.

Northern blot

A 5 μg sample of total RNA was fractionated on a 1.5% formaldehyde-agarose gel in morpholinopropanesulfonic acid (MOPS) buffer and blotted to Nytran N membrane (Schleicher & Schuell, Keene, NH, USA) with 10 × standard saline citrate (SSC) as the transfer solution. The RNA was cross-linked to the filters by UV radiation. The filter was hybridized in the hybridization buffer (Sigma, St. Louis, USA) with a ^{32}P-labeled probe at 68 °C overnight. The hot filter was then washed and exposed to X-ray film. Equal amounts of RNA loading were confirmed by ethidium bromide staining before blotting.

Data acquisition and analysis

The Microarray Suite (MAS) 5.0 (Affymetrix, Santa Clara, CA) was used to assign present call (P<0.05) or absent call (P > 0.065) for each probe set. Because there were three replicates, the marginal call ($0.065 \geqslant P \geqslant 0.05$) was reassigned as present call. Over 85% of the probe sets assigned as present call had present calls in all three replications. A probe set with present (or absent) calls in two of the three replicates was assigned as present (or absent) calls for the treatment. GeneChip RMA (GCRMA) provided in GeneSpring (Agilent Technologies, Palo Alto, CA) was chosen to determine the signal intensity for probe sets for further analysis (Wu and Irizarry 2004). The probe sets that satisfied one of the following standards were selected to identify GA- or ABA-responsive genes by SAM (Tusher et al. 2001): (i) probe sets assigned as present calls in all treatments; (ii) probe sets assigned as present calls in the control had a stronger average signal in the control than in treatments; and (iii) genes assigned as present calls in treatments had a stronger average signal in the treatment than control. The original data will be available in the public domain.

BarleyBase (http://barleybase.org/) was used for gene ontology analysis (Shen et al. 2005). The HarvEST: Barley (version 1.35; http://harvest.ucr.edu/), Munich Information Center for Protein Sequence (Schoof et al. 2004), and Universal Protein Resource (Bairoch et al. 2005), were used to conduct gene functional annotation in addition to manually editing. An E score of $1\times E^{-20}$ of BLAST-X between a barley sequence and Arabidopsis sequence in HarvEST : Braley was used as a cutoff to define the homologous gene. The *Arabidopsis* homologues of the GA- and ABA-response genes were imported into MapMan (version 1.4.3, http://gabi.rzpd.de/projects/MapMan) for further function analysis (Thimm et al. 2004). In these processes, 506, 440, 90, and 42 homologs were found for GA up-, GA down-, ABA up- and ABA down-regulated genes, respectively. In addition, BarleyBase was specifically used in the search for hydrolase genes.

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Note: Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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